

**REMARKS**

Applicants submit that pending claims 1, 9-13, 15, 16, 22-32, and 34-38 are in condition for allowance, or are in better condition for presentation on appeal, and respectfully request that the claims as amended be entered.

**Rejections Under 35 U.S.C. §112, First Paragraph**

Claims 1, 9-13, 15, 16, 22-32, and 34-38 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking written description support.

Applicants traverse the rejection for the reasons given below.

The Office Action alleges, in pertinent part, that the specification does not disclose “adhesion to a defined substrate lacking a feeder layer.” Further, the Action intimates that the phrase “lacking telomerase activity” does not meet the standard for adequate written description. Applicants respectfully submit that such allegations are incorrect.

Applicants offer the following paragraphs to support the position that the terms at issue meet the written description requirement. For “defined substrates”:

[0064] “In another embodiment and use of the invention, EBD cells are used to optimize the *in vitro* growth and culture conditions for maintaining an undifferentiated state, or, for differentiating the cells. High-throughput screens can be established to assess the effects of media components, exogenous growth factors, and **attachment substrates**. These substrates include viable cell feeder layers, cell extracts, defined extracellular matrix components, substrates which **promote three-dimensional growth** such as methylcellulose and collagen, novel cell attachment molecules, and/or matrices with growth factors or other signaling molecules embedded within them. This last approach may provide the spatial organization required for replication of complex organ architecture (as reviewed in Saltzman, *Nature Medicine* 4:272-273, 1998).” (p. 20) (emphasis added);

[0099] “As discussed above, production of cells, tissues and organs for transplantation may require combinations of genetic modifications, *in vitro* differentiation, and **defined substrate** utilization of the cells of the invention to generate the desired altered cell phenotype and, if a tissue or organ is to be generated, the necessary **three-dimensional architecture required** for functionality. For example, a replacement organ may require vasculature to deliver nutrients, remove waste products, and deliver products, as well as specific cell-cell contacts. A diverse cell population will be required to carry out these and other specialized functions, such as the capacity to repopulate by lineage-restricted stem cells.” (p. 33, bridging to p. 34) (emphasis added);

[0134] “Additional methods employ specialized media and growth substrates to enhance, select or direct the growth of particular cell types. For example, the culture media can comprise reduced serum; or, it can be serum-free, in order to fully define and control the growth processes and avoid the uncontrolled effects of serum components. These uncontrolled effects can be due to the presence of a wide and variable variety of growth factors known to be present in serum. Some examples of serum-free media or reduced serum media are EGM2mv (Clonetics), various muscle-specific growth media and hepatocyte maintenance media (Hepatostim, Collaborative BioAlliance, Stony Brook, NY). Some additional examples of adhesion matrices are human extracellular matrix extract (hECM) and Matrigel3 (Collaborative Biomedical Products), laminin, poly(L)-ornithine and **fibronectin**. Growth media can be further enhanced with a wide variety of compounds including, but not limited to, retinoic acid, dimethylsulfoxide (DMSO), cAMP elevators such as forskolin, isobutylmethylxanthine, and dibutyl cAMP, cytokines such as basic fibroblast growth factor, epidermal growth factor, platelet derived growth factor (PDGF and PDGF-AA) nerve growth factor, T3, sonic hedgehog (Shh or N-Terminal fragment), ciliary neurotrophic factor

(CNTF), erythropoietin (EPO) and bone morphogenic factors.” (p.46 bridging to p.47) (emphasis added);

[0138] “RPMI growth media comprised RPMI 16403 (LTI), 15% FCS, non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, EGM2mv media (which contains 5% FCS), hydrocortisone, hbFGF, hVEGF, R<sup>3</sup>-IGF-1, ascorbic acid, hEGF, heparin, gentamycin, amphotericin. Hepatostim media (Becton Dickinson), which is a modified Williams E medium supplemented with 10ng/ml hEGF, 1 µM dexamethasone, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA, 5.35 µg/ml linoleic acid, 2 mM L-glutamate. ITSFn media (*see, e.g.,* Brustle, *Science* 285:754, 1999), which is DMEM/F12 supplemented with 5 µg/ml insulin, 50 µg/ml transferrin, 30 mM selenium chloride, 5µg/ml fibronectin. Matrices included bovine collagen I (Collaborative Bioscience) at 10 µg/cm<sup>2</sup>, and human extracellular matrix (Collaborative Bioscience) at 5 µg/cm<sup>2</sup>. Cells were cultured at 37°C, 5% CO<sub>2</sub>, 95% humidity and routinely passaged at 1:10 to 1:40 by using 0.05% trypsin, 0.53 EDTA (LTI), for 5 min., 37°C. Low serum and serum free media cultures were treated with trypsin inhibitor (Clonetics) and spun down and resuspended in growth media. Cells were cryopreserved in the presence of 40% FCS, 10% DMSO in a controlled rate freezing vessel, and stored in liquid nitrogen. The cultures LV, SL, LU2 were derived from EG cell cultures initiated from 7, 8 and 9 week LMP gonadal ridge tissue.” (p. 47, bridging to p. 48) (emphasis added);

[0143] “Individual growth environments produced different gene expression patterns. The EGM2MV/collagen I environment that produced the highest rate of cell proliferation resulted in a culture containing cells that also express CD34. Expression of the other markers varied with culture conditions and genotype. For instance, in the LV culture, the endoderm marker GATA4 was expressed only

when grown in the EGM2MV/collagen I environment, while in the SL culture, all environments results in GATA4 expression. AC133 (a cell surface marker of hematopoietic stem cells) had weak or absent expression in all conditions except RPMI/collagen and to a lesser extent RPMI/hECM in the SL culture.” (p. 49) (emphasis added); and

[0151] “Often cells in Matrigel3 shift from a proliferative form to a more differentiated and non-proliferative one. In order to investigate whether the EBD cells of the invention were capable of proliferating in Matrigel3, several EBs or “nests” were collected and disaggregated by digestion in dispase. Disaggregated single cells that were re-plated into a thick layer of Matrigel3 slowly formed the highly branched and extended morphology seen in cells of the original “nests.” However, in one month, these single cells did not form independent complex structures or seem to have proliferated. If the Matrigel3 disaggregated cells were plated onto thin layers of collagen I, glass or plastic, they reverted to an adherent cell morphology and did not proliferate extensively.” (p. 52 bridging to page 53) (emphasis added).

Clearly, a chemically described surface upon which cells are grown would meet the definition of defined substrate (see, e.g., <http://helios.bto.ed.ac.uk/bto/glossary/s.htm> and <http://library.thinkquest.org/C001764/glossary/s.htm>). Further, Applicants offer Exhibit A (Arai et al., Blood (1999) 93(11):3713-3722), at p. 3715, col. 1, which states for example:

“To confirm that overexpression of CrKL activates cell adhesion, 32DE/Tet-CrKL cells as well as 32DE/TA cells were cultured with or without tetracycline for 24 hours and allowed to attach to wells coated with a defined substrate, fibronectin, for 30 minutes . . .”

Moreover, as stated in Fujikawa v. Wattanasin, 39 U.S.P.Q2d 1895 (Fed. Cir. 1996), “*ipsis verbis* disclosure is not necessary to satisfy the written description requirement of section 112. Instead, the disclosure need only reasonably convey to persons skilled in the art that the inventor had possession of the subject matter in question.”

Applicants submit that in view of a) these explicit statements from the specification, b) the evidence that the art recognizes that a “defined substrate” would include an extracellular matrix component (e.g., fibronectin), and c) the case law, one of skill in the art would reasonably conclude that Applicants were in possession of a “defined substrate” as claimed.

Regarding the element “lacking telomerase activity,” Applicants offer the following express citation:

[0180] “To determine the proliferative capacity of EBD cultures, LVEC, SLEC, LU2EC and SDEC were continuously passaged. After approximately 70-80 PD, these cultures failed to divide. Continuous passage of cultures in environments less favorable to proliferation has not been carried out; however, most EBD cultures are capable of at least 40 PD. To determine whether the proliferation of EBD cultures may be limited by the absence of telomerase, telomeric repeat amplification protocol assays were performed on LVEC and SDEC cultures that had undergone approximately 20 PD after EBD cell establishment. Telomerase assays were performed by using a telomeric repeat amplification protocol followed by ELISA detection of amplified products (TeloTAGGG PCR ELISA PLUS3, Roche). **No telomerase activity was detected in either culture**, consistent with the hypothesis that cell division in the absence of telomerase activity leads to cellular senescence.” (p. 66 bridging to p. 67) (emphasis added).

As such, one of skill in the art could envision the properties of growth on “defined substrate” and “lacking of telomerase activity” as claimed, and would appreciate that the inventors were in possession of the genus as claimed at the time the invention was filed. That is all that is required.

For these reasons, Applicants respectfully request that the rejection be withdrawn.

Claims 1-9-13, 15, 16, 22-32, and 34-38 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement.

Applicants traverse the rejection for the reasons given below.

The Office Action alleges, in pertinent part, that the specification “does not enable a use for EBD cells lacking telomerase activity that is senescent cells. The uses disclosed in the specification each require the cells to actively divide. Senescent cells are not going to be useful for cell culture, tissue transplantation, tissue engineering, drug discovery or gene therapy. A senescent cell has quit dividing. Each disclosed use requires that the cells divide.” Applicants respectfully submit that the position in the Action is incorrect.

As stated in Engel Industries, Inc. v. Lockformer Co., 20 U.S.P.Q.2d 1300 (Fed. Cir. 1991), “[t]he enablement requirement is met if any description enables any mode of making and using the claimed invention.” Applicants submit that senescent cells can be used for transplantation. In support of this position Applicants offer Exhibit B: Ostenfeld et al., Human Neural Precursor Cells Express Low Levels of Telomerase in Vitro and Show Diminishing Cell Proliferation with Extensive Axonal Outgrowth following Transplantation. *Experimental Neurology* (2000) 164(1):215-226. The Abstract clearly shows that a) human neural precursor cells express very low levels of telomerase at early passage, that decreases to undetectable levels at later passages; b) these cell when implanted, fiber outgrowth continued, even though there were no dividing cells in the graft; and c) for Parkinson’s disease, because such cells afford a good safety profile, they “may provide the ideal basis for the repair of other lesions of the CNS where extensive axonal outgrowth is required.”

The specification explicitly states:

**[0007]** “The invention is directed to novel cells that are derived from human embryoid bodies (EBs), which are in turn produced by culturing EG cells. Such embryoid body derived (EBD) cells and cell lines are relatively uncommitted or progenitor cells.

EBD cells, while not immortal, display robust and long-term proliferation in culture

with a normal karyotype and can be cryopreserved and cloned. They can be efficiently transfected with retroviruses and lentivirus, for example, and can be genetically manipulated. Although EBD cells have a developmentally broad multilineage expression profile and do not form tumors (*e.g.*, differentiated embryonic tumors or teratomas) when injected *in vivo*, such as into severe combined immunodeficiency (SCID) mice. As a result, EBD cells have a variety of uses, for example, in transplantation therapies for the treatment of such diseases as Parkinson's disease, amyotrophic lateral sclerosis (ALS), stroke, injury to motor neurons, including spinal cord injury, and diabetes." (p. 2 bridging to page 3) (emphasis added).

Therefore, because a) transplantation therapies for the treatment of such diseases as Parkinson's disease is expressly recited in the specification as filed; b) the art recognizes the use of senescent cells in transplantation therapies for the treatment of Parkinson's disease; and c) the specification enables a mode of making and using the claimed invention (*e.g.*, EBD cells lacking telomerase activity), the claims are enabled. Thus, the specification provides appropriate guidance and working examples such that one of skill in the art could practice the invention as claimed, in the absence of undue experimentation. That is all that is required.

For these reasons, Applicants respectfully request that the rejection, including as it may be applied to the amended claims, be withdrawn.

In re Application of:  
Shamblott and Gearhart  
Application No.: 09/767,421  
Filing Date: January 22, 2001  
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PATENT  
Attorney Docket No. JHU1750-1


**Conclusion**

Applicants submit that pending claims 1, 9-13, 15, 16, 22-32, and 34-38 are in condition for allowance, or are in better condition for appeal. The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to this submission.

A check in the amount of \$450.00 is enclosed to cover a Two Month Extension of Time fee. No other fee is deemed necessary with the filing of this paper. However, the Commissioner is hereby authorized to charge any fees required by this submission, or credit any overpayments, to Deposit Account No. 07-1896 referencing the above-identified docket number. A copy of the Transmittal Sheet is enclosed.

Respectfully submitted,

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## CrkL Activates Integrin-Mediated Hematopoietic Cell Adhesion Through the Guanine Nucleotide Exchange Factor C3G

By Ayako Arai, Yurika Nosaka, Hitoshi Kohsaka, Nobuyuki Miyasaka, and Osamu Miura

CrkL is a member of the Crk family of adapter proteins consisting mostly of SH2 and SH3 domains. CrkL is most abundantly expressed in hematopoietic cells and has been implicated in pathogenesis of chronic myelogenous leukemia. However, its function has not been precisely defined. Here, we show that overexpression of CrkL enhances the adhesion of hematopoietic 32D cells to fibronectin. The CrkL-induced increase in cell adhesion was blocked by antibodies against VLA-4 ( $\alpha 4\beta 1$ ) and VLA-5 ( $\alpha 5\beta 1$ ) but was observed without changes in surface expression levels of these integrins. Studies using CrkL mutants demonstrated that the SH2 domain is partially required for enhancing cell adhesion, whereas the C-terminal SH3 domain as well as the tyrosine phosphorylation site (Y207) is dispensable. In contrast, the N-terminal SH3 domain, involved in binding C3G

and other signaling molecules, was showed to play a crucial role, because a mutant defective of this domain showed an inhibitory effect on the cell adhesion to fibronectin. Furthermore, overexpression of C3G also increased the adhesion of hematopoietic cells to fibronectin, whereas a C3G mutant lacking the guanine nucleotide exchange domain abrogated the CrkL-induced increase in cell adhesion. On the other hand, a dominant negative mutant of H-Ras or that of Raf-1 enhanced the basal and CrkL-induced cell adhesion and that of R-Ras modestly decreased the adhesion. Taken together, these results indicate that the CrkL-C3G complex activates VLA-4 and VLA-5 in hematopoietic cells, possibly by activating the small GTP binding proteins, including R-Ras, through the guanine nucleotide exchange activity of C3G.

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HEMATOPOIESIS takes place in close contact with the bone marrow microenvironment, which is composed of stromal cells and extracellular matrix components, including fibronectin. Members of the integrin superfamily of adhesion molecules mainly mediate adherence of hematopoietic cells to both the extracellular matrix and stromal cells. Integrins are heterodimers of  $\alpha$  and  $\beta$  subunits that can pair to form more than 20 receptors.<sup>1,2</sup> Integrins of the  $\beta 1$  subfamily, mostly VLA-4 ( $\alpha 4\beta 1$ ) and VLA-5 ( $\alpha 5\beta 1$ ), have been identified on most of the hematopoietic progenitor cells as well as on various hematopoietic cell lines<sup>3-5</sup> and shown to bind ligands especially when these cells are stimulated with growth-stimulating cytokines such as interleukin-3 (IL-3), granulocyte-monocyte colony-stimulating factor, erythropoietin (Epo), and stem cell factor.<sup>6-9</sup> One of the ligands involved in the adhesion of hematopoietic cells through VLA-4 and VLA-5 is fibronectin, which preferentially mediates adhesion of primitive progenitor cells to the bone marrow microenvironment.<sup>10</sup> Recently, accumulating evidence has suggested that adhesive interaction mediated by integrins of  $\beta 1$  subfamily and fibronectin plays a critical role in controlling proliferation, apoptosis, migration, and mobilization of hematopoietic cells.<sup>4,8,11-18</sup> Thus, knowledge of the mechanisms by which the functional states of these integrins are regulated is critical to our understanding of the physiologic mechanisms responsible for the regulation of normal hematopoiesis.

The Crk proteins, originally identified as homologues of the product of the *v-crk* oncogene,<sup>19</sup> are adapter proteins composed of SH2 and SH3 domains with very short intervening sequences. Three forms of cellular Crk proteins have been found; both Crk II and CrkL (for Crk-like) have the domain structure SH2-SH3-SH3, although Crk I, the alternatively spliced form of Crk II, lacks the C-terminal SH3 domain.<sup>20,21</sup> The N-terminal SH3 domain of CrkL has been shown to bind Sos1 and C3G, two guanine nucleotide exchange proteins for the Ras family of small GTPases.<sup>22,23</sup> Interestingly, recent studies have established that CrkL, which is most abundantly expressed in hematopoietic cells,<sup>24</sup> also binds through its N-terminal SH3 domain to the BCR-ABL fusion protein expressed in chronic

myelogenous leukemia cells and becomes phosphorylated at Y-207.<sup>25-28</sup> CrkL also becomes tyrosine phosphorylated in hematopoietic cells stimulated with stem cell factor,<sup>29</sup> thrombopoietin,<sup>30</sup> Epo,<sup>31</sup> IL-3,<sup>31</sup> and IL-2.<sup>32</sup> In addition, CrkL, through its SH2 domain, forms complexes with tyrosine-phosphorylated signaling molecules, including c-Cbl,<sup>29,31-33</sup> Shc,<sup>31</sup> and SHP-2<sup>31</sup> in hematopoietic cells stimulated with cytokines. Thus, it is implied that CrkL may play a role in growth control and leukemic transformation of hematopoietic cells. However, the function of CrkL in hematopoietic cells has not been precisely defined. In the present study, we show that overexpression of CrkL activates adhesion of hematopoietic cells to fibronectin through VLA-4 and VLA-5. CrkL was further shown to transduce the signal to activate these integrins through the guanine nucleotide exchange activity of C3G.

### MATERIALS AND METHODS

**Cells and reagents.** A clone of IL-3-dependent 32D cells expressing the wild-type murine Epo receptor (32D/EpoR-Wt) was previously described<sup>34</sup> and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1 U/mL human recombinant Epo. COS7 cells were cultured in Dulbecco's modified Eagle medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS. Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan). Recombinant murine IL-3 was purchased from Pepro-Tech Inc (Rocky Hill, NJ).

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Antibodies against CrkL, C3G, and R-Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies (MoAbs) against murine  $\alpha 4$  (428) and  $\alpha 5$  (5H10-27) integrin subunits were from Seikagaku Corp. (Tokyo, Japan) and from PharMingen (San Diego, CA), respectively. Fluorescein (DTAF)-conjugated goat anti-rat IgG secondary antibody was obtained from Immunotech (Marseille, France). Human plasma fibronectin was purchased from GIBCO-BRL (Grand Island, NY).

**Expression plasmids.** An expression plasmid for human CrkL, pSG-CrkL,<sup>35</sup> was kindly provided by Dr John Groffen (Childrens Hospital Los Angeles, Los Angeles, CA). Expression plasmids for various CrkL mutants were constructed by deletion of the following fragments from the CrkL cDNA: dSH2, a Cfr101 fragment (nucleotides 560 to 752); dSH3N, a *DraI*-*AluI* fragment (nucleotides 941 to 1049); dY, an *AvaIII*-*PstI* fragment (nucleotides 1127 to 1163); dSH3C, an *AvaIII*-*BalI* fragment (nucleotides 1127 to 1327).

For construction of pTet-CrkL, in which the CrkL cDNA is placed downstream of a tetracycline operator (*tetO*)-controlled promoter, a 5' portion of the CrkL cDNA (nucleotides 514 to 844) was amplified by the polymerase chain reaction (PCR) with 5' and 3' primers of 5'-CCGGATCCTCCGCCAGGTTTCGACTC-3' and 5'-CCGAATTCATCCCATTGGTGGGCTTGGAT-3', respectively. The primer sequences were designed to add the *Bam*HI and *Eco*RI recognition sequences at the 5' and 3' ends, respectively, of the amplified fragment. These sites were then used for subcloning of the amplified fragment into the multiple-cloning site of an expression plasmid, pJ3H,<sup>36</sup> obtained from American Type Culture Collection (Rockville, MD). The *SalI*-*Clal* fragment, encompassing the amplified region, was then excised from this plasmid and subcloned between the *SalI* and *Clal* sites of pTet-Splice (GIBCO-BRL). This plasmid was then digested with *CpoI* and *EcoRV* to subclone the *CpoI*-*BglII* fragment, containing nucleotides 536 to the 3' end of the CrkL cDNA, from pSG-CrkL to replace the PCR-amplified region, thus giving pTet-CrkL.

An expression plasmid for C3G, pcDNA-C3G, was constructed by subcloning the *HindIII*-*Bam*HI fragment (nucleotides 66 to 3377) of C3G cDNA,<sup>22</sup> obtained through the Riken Gene Bank (Ibaraki, Japan) with the permission from Dr Michiyuki Matsuda (National Institute of Health, Tokyo, Japan), into pcDNA3 (Invitrogen, San Diego, CA). An expression plasmid for mutant C3G, pcDNA-C3G-dSS, was created by deletion of the *SmaI*-*ScaI* fragment of C3G cDNA (nucleotides 2609 to 2999) from pcDNA-C3G. Tetracycline responsive expression plasmid for C3G and the C3G mutant, pTet-C3G and pTet-C3G-dSS, were constructed by subcloning the *HindIII*-*AvrII* fragments (nucleotides 66 to 3360) from pcDNA-C3G and pcDNA-C3G-dSS, respectively, between the *HindIII* and *SpeI* sites of pTet-Splice.

For construction of an expression plasmid for mutant Raf-1, pcDNA-Raf-dSE, the Raf-1 cDNA was excised from p627,<sup>37</sup> obtained from the Riken Gene Bank, by digestion with *EcoRI* and *XbaI* and subcloned into pcDNA3 to give pcDNA-Raf-1. The *StuI*-*EcoRV* fragment (nucleotides 1122 to 2028) was then deleted to give pcDNA-Raf-dSE. An expression plasmid for a dominant negative mutant of R-Ras, pcDNA-R-Ras43N,<sup>38</sup> was kindly provided by Dr Erkki Ruoslahti (La Jolla Cancer Research Center, La Jolla, CA). An expression plasmid for dominant negative H-Ras, pcDNA-H-Ras17N, was constructed by subcloning cDNA coding for H-Ras17N (Upstate Biotechnology, Lake Placid, NY) into the pcDNA3 vector.

**Transfection.** Transfection for stable expression was performed essentially as described previously.<sup>34</sup> In brief, 32D/EpoR-Wt cells were transfected with or without 5  $\mu$ g of pTet-CrkL along with 5  $\mu$ g of pTet-tTak (GIBCO-BRL), which is an expression plasmid for the tetracycline transactivator (tTA),<sup>39</sup> and 1  $\mu$ g of pSV-Zeo (Invitrogen) by electroporation at 960  $\mu$ F and 300 V, followed by selection in medium containing Zeocine (Invitrogen) and 500 ng/mL tetracycline. Six clones transfected with pTet-CrkL were isolated by limiting dilution and

examined for the induction of CrkL expression by anti-CrkL immunoblotting of cell lysates prepared after withdrawal from tetracycline for 24 hours. The clone inducibly expressing the highest level of CrkL, 32DE/Tet-CrkL, was selected for the subsequent studies. Clones transfected with pTet-tTak and pSV-Zeo alone were similarly selected and examined for the expression of tTA by the luciferase assay by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), the pUHC13-3 (GIBCO-BRL) and a control Renilla luciferase plasmid, pRL-SV (Promega), a reporter plasmid, as described previously.<sup>40</sup> The clone inducibly expressing the highest level of tTA, which was comparable with that expressed by 32DE/Tet-CrkL, was designated as 32DE/tTA and used for the subsequent studies. 32DE/Tet-C3G and 32DE/Tet-C3G-dSS clones were similarly obtained by transfecting pTet-C3G and pTet-C3G-dSS, respectively, into 32DE/tTA cells along with pMAM2-BSD<sup>41</sup> (Funakoshi, Tokyo, Japan) followed by selection in medium containing blasticidin-S (Funakoshi).

Transfection of expression plasmids into COS7 cells was performed with the Lipofectamin reagent (GIBCO-BRL), as described previously.<sup>42</sup> Cells were harvested for analysis with immunoprecipitation and immunoblotting 2 days after transfection.

**Immunoprecipitation and immunoblotting.** Cells were solubilized with a lysis buffer composed of 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL aprotinin, and 10  $\mu$ g/mL leupeptin. Cell lysates were subjected to immunoprecipitation and immunoblotting as described previously.<sup>34</sup>

**Cell adhesion assays.** For stable transfectants, cell adhesion assay was performed essentially as described with some modifications.<sup>38</sup> In brief, 96-well, flat-bottom tissue culture plates were coated with indicated concentrations of fibronectin overnight at 4°C. Plates were then blocked with 1% bovine serum albumin (BSA) at 37°C for 1 hour followed by washing three times with RPMI 1640 containing 0.2% BSA, referred to as cell adhesion medium. Cells were washed twice and resuspended in cell adhesion medium supplemented with 5 ng/mL IL-3, unless indicated otherwise. Cells (5 to 10  $\times 10^4$ /well) were added to each well in triplicate and incubated for 30 minutes at 37°C. In some experiments, cells were incubated with indicated concentrations of anti-integrin antibodies or irrelevant MoAb for 15 minutes at room temperature before plating on fibronectin coated wells. Plates were then washed three times with cell adhesion medium to remove unbound cells. Cells remaining attached to the plates were measured by the sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric assay (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's recommendation. After subtraction of background cell binding to BSA-coated wells, the percentage of adherent cells was determined by dividing the optical density of the adherent cells by that of the initial cell input.

For adhesion assay of transiently transfected cells, 32D/EpoR-Wt cells were electroporated at 960  $\mu$ F and 300 V with indicated amounts of relevant plasmids and 1  $\mu$ g of a control Renilla luciferase plasmid, pRL-SV. After a recovery period of 1 day, cells were subjected to the cell adhesion assay described above except that 4  $\times 10^5$  cells were plated on fibronectin-coated 24-well plates in duplicate, and the adhesion was assayed by the luciferase activity of cell lysates.

All the cell adhesion assays in Results were repeated at least three times, and the results were reproducible.

**Flow cytometry.** To analyze the surface expression of VLA-4 and VLA-5, 32DE/Tet-CrkL cells were cultured in the presence or absence of tetracycline for 24 hours and stained with anti- $\alpha 4$  or anti- $\alpha 5$  antibody or left unstained as control. Cells were further stained with fluorescein-labeled secondary antibody and analyzed with an Epics Elite flow cytometer (Coulter Electronics, Miami, FL).

## RESULTS

*Overexpression of CrkL increases adhesion of cells to fibronectin.* To explore the functions of CrkL in hematopoietic cells, we established a clone of 32D/EpoR-Wt cells, 32DE/Tet-CrkL, which overexpresses CrkL when withdrawn from tetracycline, as described in Materials and Methods. As shown in Fig 1A, 32DE/Tet-CrkL cells, which grow in suspension, became highly adherent when cultured in the absence of tetracycline and extended long protrusions. 32DE/TA cells, which express only tTA at a comparable level with 32DE/Tet-CrkL when withdrawn from tetracycline, did not show this change. To confirm that overexpression of CrkL activates cell adhesion, 32DE/Tet-CrkL cells as well as 32DE/TA cells were cultured with or without tetracycline for 24 hours and allowed to attach to wells coated with a defined substrate, fibronectin, for 30 minutes in the presence of IL-3. As shown in Fig 1B, 32DE/Tet-CrkL cells attached dramatically better to fibronectin when tetracycline was removed from culture medium, whereas 32DE/TA cells, cultured with or without tetracycline, attached poorly to this substrate. Anti-CrkL immunoblotting of lysates obtained from cells cultured under the same conditions confirmed that CrkL was overexpressed in 32DE/Tet-CrkL cells in the absence of tetracycline (Fig 1B, upper panel). When removed from tetracycline to overexpress CrkL, 32DE/Tet-CrkL cells were also shown to attach better to wells coated with various concentrations of fibronectin (Fig 1C). In accordance with previous reports,<sup>6,9</sup> 32DE/Tet-CrkL cells starved from Epo and IL-3 for 16 hours barely attached to fibronectin, whereas the adhesion was remarkably activated when cultured in the presence of IL-3 (Fig 1D). Although Epo also activated the adhesion of 32DE/Tet-CrkL or 32D/EpoR-Wt cells in repeated experiments, the Epo-induced increase in cell adhesion was always only moderate and much less than that induced by IL-3 (Fig 1D; data not shown). As shown in Fig 1D, the overexpression of CrkL induced by withdrawal from tetracycline dramatically increased the low adhesion levels of 32DE/Tet-CrkL cells starved from cytokines or cultured in Epo, while the IL-3-induced, high level of adhesion was only moderately increased by the CrkL overexpression.

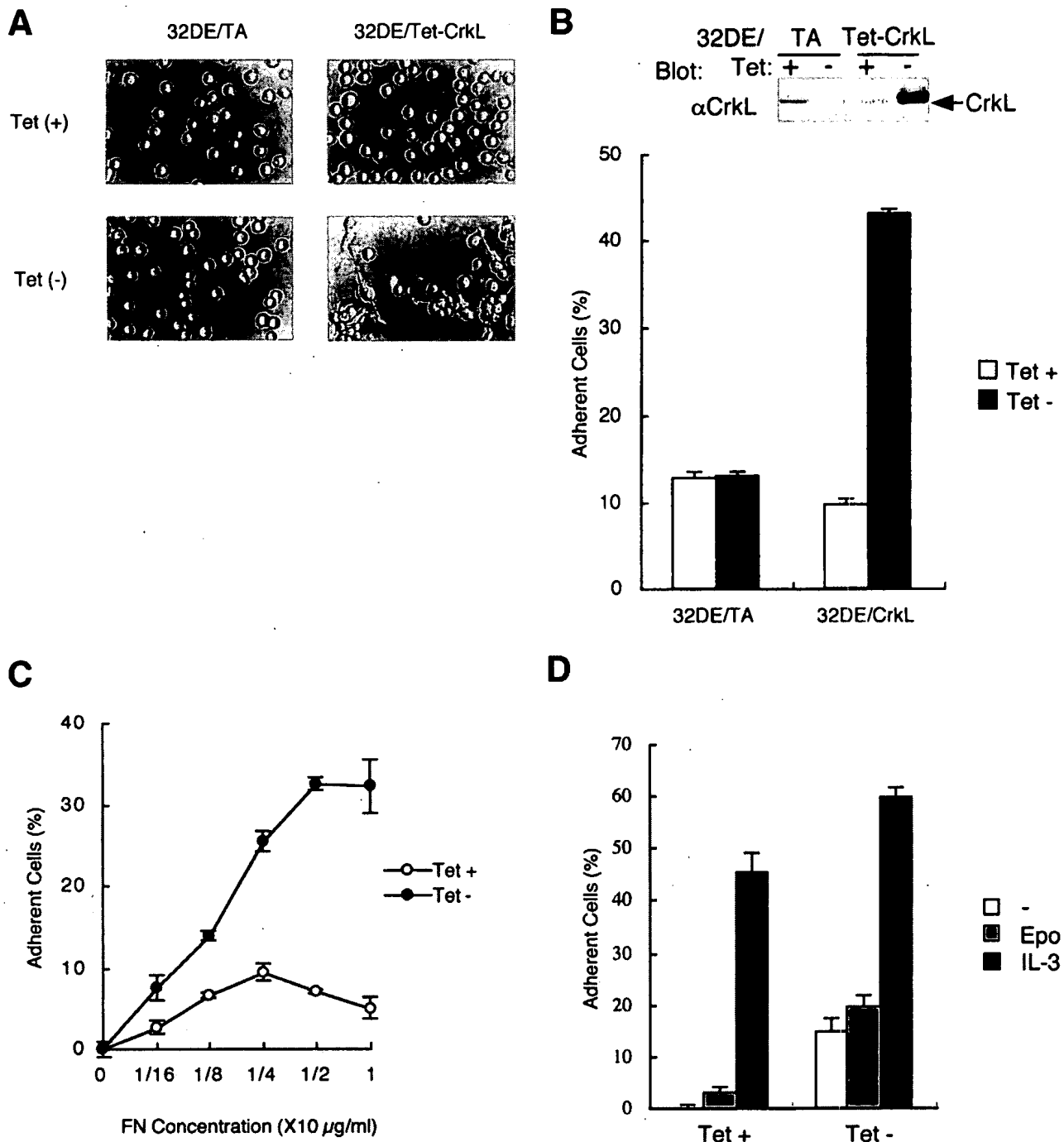
*CrkL increases cell adhesion to fibronectin by activating VLA-4 and VLA-5.* Integrins of the  $\beta 1$  family, mostly VLA-4 ( $\alpha 4\beta 1$ ) and VLA-5 ( $\alpha 5\beta 1$ ), have been shown to mediate adhesion of hematopoietic cells, including 32D cells<sup>38</sup> to fibronectin. Therefore, to identify the receptors involved in the CrkL-induced increase of cell adhesion to fibronectin, we examined the effects of antibodies against VLA-4 and VLA-5 on adhesion of CrkL-overexpressing cells to fibronectin. As shown in Fig 2A, a function-blocking anti- $\alpha 4$  or anti- $\alpha 5$  integrin antibody, when added alone, partially inhibited the adhesion of 32DE/Tet-CrkL cells cultured without tetracycline to fibronectin. Notably, when the two antibodies were added in combination, adherent cells were reduced to less than 5% of the total cells added to fibronectin-coated wells. These results agree with the previous report<sup>38</sup> that 32D cell attachment to fibronectin is mediated by VLA-4 and VLA-5 integrins and further indicate that overexpression of CrkL enhanced 32D cell attachment to fibronectin through these integrins. As shown in Fig 2B, the flow cytometry analysis using anti- $\alpha 4$  and anti- $\alpha 5$  antibodies confirmed the presence of these integrins on the cell surface

of 32DE/Tet-CrkL cells and further showed that the expression levels of these integrins were not significantly altered by withdrawal from tetracycline. Thus, these results suggest that CrkL enhances cell adhesion by increasing the activities of VLA-4 and VLA-5.

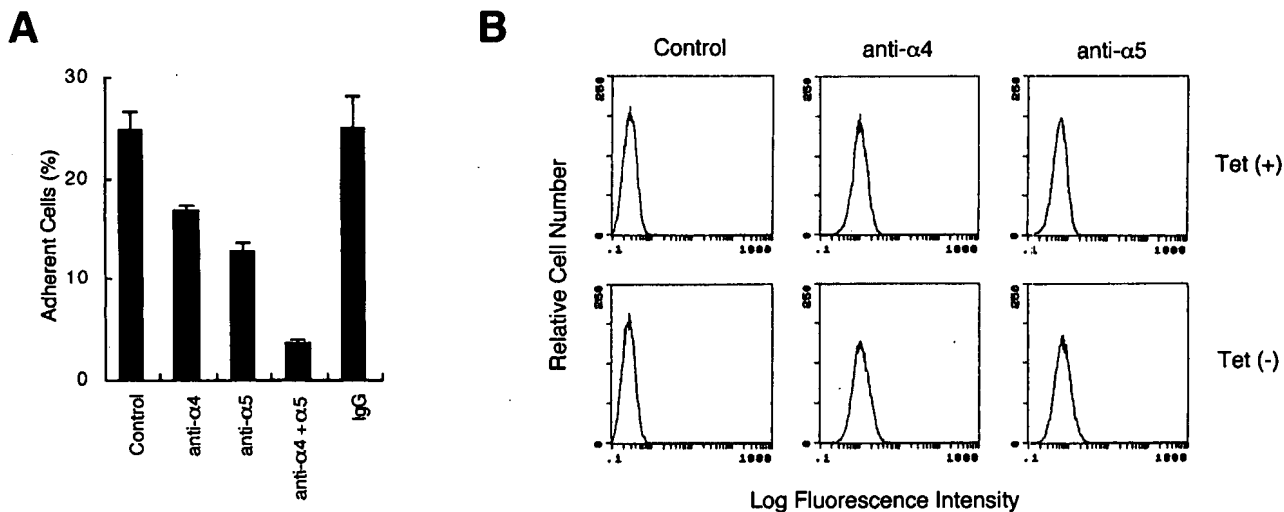
*The N-terminal SH3 domain of CrkL plays a critical role in enhancement of cell adhesion.* To explore the mechanisms by which CrkL increases the activity of VLA-4 and VLA-5, we examined the functional significance of each CrkL domain for the enhancement of cell adhesion. For this purpose, we constructed expression plasmids for CrkL mutants shown in Fig 3A. These mutants were first expressed along with C3G in COS7 cells. As shown in Fig 3B, anti-CrkL immunoblotting of transfected COS7 cell lysates showed that the CrkL mutants with expected sizes were expressed at comparable levels. Furthermore, anti-CrkL immunoblotting of anti-C3G immunoprecipitates confirmed that C3G bound all the CrkL mutants except the dSH3N mutant, which has a deletion in the N-terminal SH3 domain involved in binding C3G (Fig 3B). These results suggest that the deletions introduced into CrkL did not significantly affect the expression level or the overall structure of mutant CrkL.

To examine the abilities of CrkL mutants to enhance the cell adhesion, each mutant was then transiently expressed in 32D/EpoR-Wt cells for the cell adhesion assay, as described in Materials and Methods. As shown in Fig 3C, the dY mutant, which lacks a 12-amino-acid region containing the site of tyrosine phosphorylation,<sup>28</sup> did not show any impairment in the ability to enhance cell adhesion. The dSH3C mutant, which lacks most of the C-terminal SH3 domain in addition to the tyrosine phosphorylation site, also showed the adhesion-enhancing ability comparable with that of wild-type CrkL. On the other hand, the dSH2 mutant, in which most of the SH2 domain is lost by deletion, showed a significantly impaired ability to enhance the 32D cell adhesion to fibronectin. However, repeated experiments (data not shown) as well as a dose-dependent experiment shown in Fig 3D confirmed that the dSH2 mutant has retained the ability, although impaired, to enhance cell adhesion. In contrast, the dSH3N mutant, lacking the significant portion of the N-terminal SH3 domain, not only failed to enhance but also significantly inhibited the attachment of 32D/EpoR-Wt cells to fibronectin in repeated experiments (Fig 3C; data not shown). The inhibitory effect of the dSH3N mutant was also demonstrated to be dose dependent (Fig 3D). These results indicate that the N-terminal SH3 domain of CrkL, through which CrkL binds C3G and other signaling molecules, plays a crucial role in integrin activation. Although the SH2 domain may also play a role in integrin activation, this domain is not crucial for this function. On the other hand, neither the tyrosine phosphorylation site nor the C-terminal SH3 domain was shown to be involved in integrin activation.

*The guanine nucleotide exchange activity of C3G is involved in CrkL-induced integrin activation.* Although the N-terminal SH3 domain has been shown to bind both C3G and Grb2, we previously showed that CrkL predominantly binds C3G in 32D cells.<sup>31</sup> In addition, it was confirmed that an increased amount of C3G was associated with CrkL in 32DE/Tet-CrkL cells when CrkL was overexpressed (data not shown). Thus, we examined whether C3G is also involved in integrin activation. As



**Fig 1. Overexpression of CrkL increases adhesion of 32D cells.** (A) Morphology of 32D cells overexpressing CrkL. A clone of 32D/EpoR-Wt cells stably transfected with the expression plasmid for tetracycline transactivator alone (32DE/TA) or a clone also transfected with pTet-CrkL (32DE/Tet-CrkL) was cultured in the presence (+) or absence (-) of 100 ng/mL of tetracycline (Tet), as indicated, for 24 hours and photographed under an inverted microscope (Olympus, Tokyo, Japan). (B) Adhesion of CrkL-overexpressing 32D cells to fibronectin. 32DE/TA and 32DE/Tet-CrkL cells were cultured in the presence (+) or absence (-) of Tet, as indicated, for 24 hours and allowed to attach to wells coated with 10 μg/mL fibronectin for 30 minutes at 37°C in the presence of IL-3. The extent of cell adhesion was quantitated as described in Materials and Methods. The data represent averages ± SD of triplicate determinations. Anti-CrkL immunoblotting of cell lysates obtained under the same conditions is also shown. (C) Effect of fibronectin concentration on adhesion of CrkL-overexpressing 32D cells. 32DE/Tet-CrkL cells, cultured with or without tetracycline, as indicated, were allowed to attach to wells coated with indicated concentrations of fibronectin for the cell adhesion assay. (D) Effect of cytokines on adhesion of CrkL-overexpressing 32D cells. 32DE/Tet-CrkL cells were cultured with or without tetracycline, as indicated, for 24 hours. During the last 16 hours, cells were cultured with 1 U/mL Epo (Epo), 5 ng/mL IL-3 (IL-3), or without any cytokine (-), as indicated. Cells were allowed to attach to wells coated with 10 μg/mL fibronectin for the cell adhesion assay in the presence or absence of cytokine, as indicated.



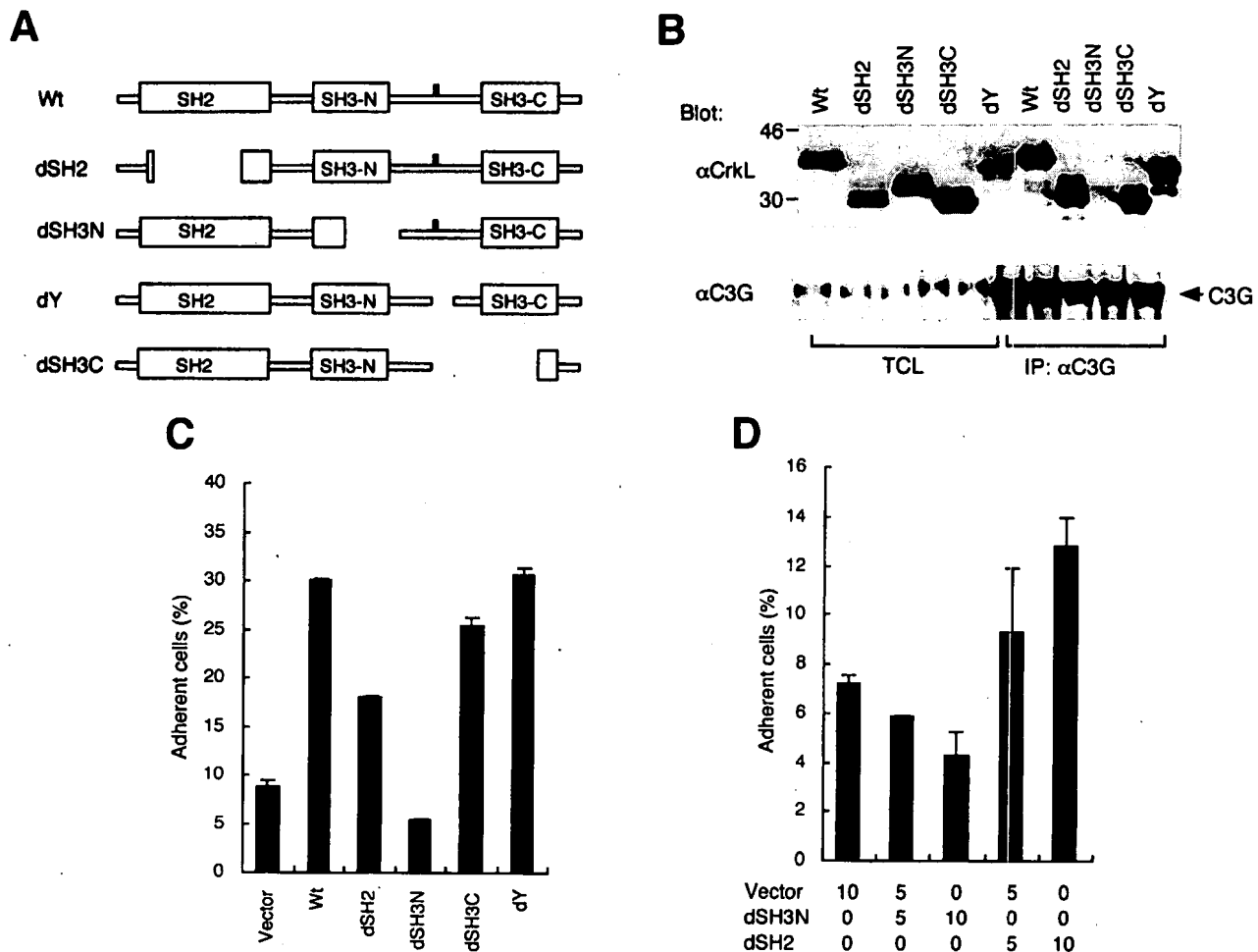
**Fig 2.** CrkL increases adhesion of 32D cells to fibronectin by activating VLA-4 and VLA-5. **(A)** Antibodies against VLA-4 and VLA-5 inhibit adhesion of CrkL-overexpressing 32D cells to fibronectin. 32DE/Tet-CrkL cells were cultured for 24 hours in the absence of tetracycline and allowed to attach to wells coated with 10  $\mu$ g/mL fibronectin in the absence (Control) or in the presence of indicated anti-integrin MoAbs or irrelevant MoAb (IgG), as indicated. The extent of cell adhesion was quantitated as described in Materials and Methods. **(B)** Analysis of VLA-4 and VLA-5 expression in 32DE/Tet-CrkL cells by flow cytometry. 32DE/Tet-CrkL cells were cultured in the presence (upper panels) or absence (lower panels) of tetracycline for 24 hours and stained with indicated anti-integrin MoAbs or left unstained as control (Control), as indicated. Cells were further stained with fluorescein-labeled secondary antibody and subjected to flow cytometry.

described in Materials and Methods, we established 32D/EpoR-Wt clones, 32DE/Tet-C3G and 32DE/Tet-C3G-dSS, which overexpress wild-type C3G and the C3G-dSS mutant, respectively, when cultured without tetracycline. When the expression level of C3G was increased by withdrawal from tetracycline (Fig 4A), 32DE/Tet-C3G cells showed a moderately increased adhesion to fibronectin, as shown in Fig 4B. In contrast, when the expression of C3G-dSS mutant, lacking the guanine nucleotide exchange domain, was induced by withdrawal from tetracycline (Fig 4A), the adhesion of 32DE/Tet-C3G-dSS cells was significantly inhibited, as shown in Fig 4B. These results indicate that the guanine nucleotide exchange activity of C3G should play a role in integrin activation.

To further confirm the involvement of C3G in CrkL-induced integrin activation, we transiently expressed wild-type C3G or the C3G-dSS mutant in 32D/EpoR-Wt cells and examined the effect on cell adhesion. As shown in Fig 5A, the transient overexpression of C3G drastically enhanced the adhesion of 32D/EpoR-Wt cells to fibronectin, thus confirming the observation in 32DE/Tet-C3G cells. When overexpressed along with CrkL, C3G further increased the cell adhesion enhanced by CrkL (Fig 5A). On the other hand, the C3G-dSS mutant drastically inhibited the adhesion of transfected cells, in accordance with the result in 32DE/Tet-C3G-dSS (Fig 5B). Importantly, when coexpressed with CrkL, the adhesion-enhancing effect of CrkL was also significantly inhibited by this mutant (Fig 5B). Taken together with the result that the C3G-binding domain of CrkL is crucial for the enhancement of cell adhesion, these data indicate that the CrkL-induced integrin activation is mediated through the guanine nucleotide exchange activity of C3G.

Because C3G activates members of the Ras subfamily of small GTP binding proteins through its guanine nucleotide

exchange activity, we next examined whether these molecules are involved in the downstream signaling pathway from the CrkL-C3G complex leading to integrin activation. First, the possible involvement of R-Ras, which activates integrin in 32D cells,<sup>38</sup> was examined. Thus, a dominant negative mutant of R-Ras, R-Ras43N,<sup>38</sup> was transiently expressed alone or along with CrkL in 32D/EpoR-Wt cells and its effect on cell adhesion was examined. In accordance with the previous report,<sup>38</sup> R-Ras43N inhibited, although modestly, the basal level of 32D/EpoR-Wt cell adhesion to fibronectin (Fig 5B). This mutant also inhibited the cell adhesion enhanced by overexpression of CrkL to a similar extent. However, the extent of inhibition induced by R-Ras43N was much less than that induced by C3G-dSS, although R-Ras43N was expressed, under the same condition, at a much higher level than that of endogenous R-Ras (data not shown). Next, the possible involvement of H-Ras was examined by using dominant negative mutants of H-Ras and Raf-1, an effector molecule of H-Ras. As shown in Fig 5C, these mutants, H-Ras17N and Raf-dSE, significantly enhanced the adhesion of 32D/EpoR-Wt cells, which is in accordance with a previous report that H-Ras and Raf-1 inhibited integrin activity.<sup>43</sup> The enhanced adhesion of cells overexpressing CrkL was also increased slightly by coexpression of these mutants (Fig 5C). These results indicate that R-Ras and H-Ras modulate the integrin activity positively and negatively, respectively, in 32D cells and raise a possibility that the CrkL-C3G complex may transduce the integrin activation signal, although partly, through activation of R-Ras. However, because the inhibitory effect of R-Ras43N on the CrkL-enhanced cell adhesion was only modest, it is speculated that other signaling molecules, most likely other small GTPases, may play more significant roles in integrin activation by CrkL and C3G.



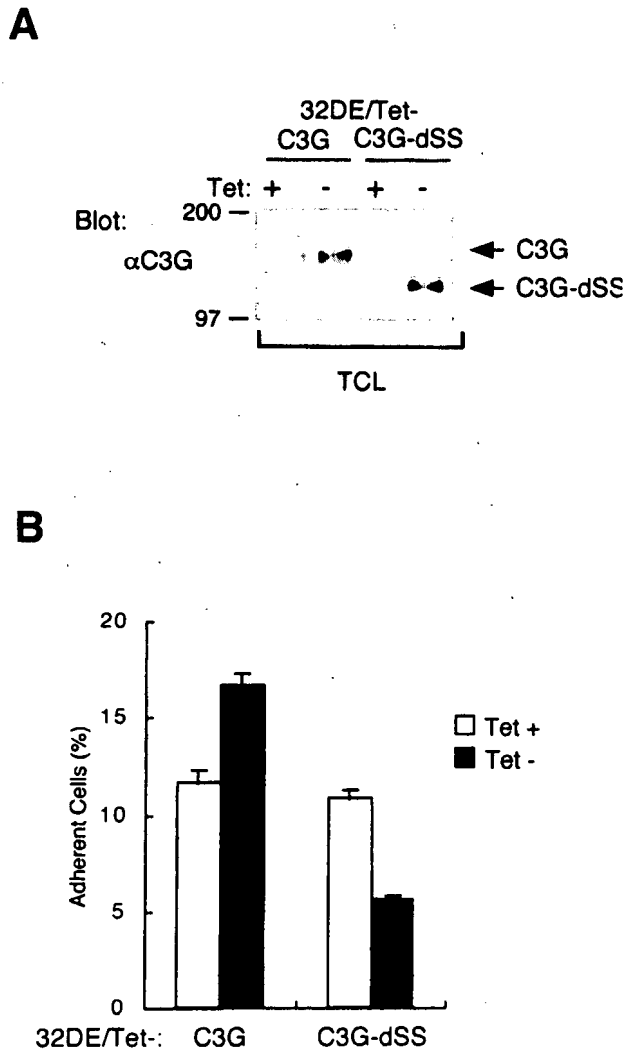
**Fig 3.** The N-terminal SH3 domain of CrkL plays a critical role in enhancement of cell adhesion. (A) Schematic representation of CrkL and its mutants. (B) Transient expression of CrkL and its mutants with C3G in COS7 cells. Expression plasmids for wild type and various mutants of CrkL, as indicated, were transfected with that of C3G into COS7 cells. Cells were harvested 2 days after transfection, and total cell lysates (TCL) and anti-C3G immunoprecipitates were subjected to anti-CrkL immunoblotting followed by reprobing with anti-C3G. (C) Effects of transiently expressed CrkL mutants on adhesion of 32D/EpoR-Wt cells to fibronectin. The expression plasmids for wild type and various mutants of CrkL, as indicated, were transfected into 32D/EpoR-Wt cells along with pRL-SV. Transiently transfected cells were subjected to the cell adhesion assay as described in Materials and Methods. (D) Dose-dependent effects of the CrkL dSH3N and dSH2 mutants on 32D/EpoR-Wt cell adhesion to fibronectin. 32D/EpoR-Wt cells were transfected with indicated amounts (microgram) of the expression plasmids for dSH3N and dSH2 mutants of CrkL or the pSG5 vector plasmid and subjected to the cell adhesion assay.

## DISCUSSION

In this study, we have showed that overexpression of CrkL activates the adhesion of hematopoietic cells to fibronectin. The enhancement of cell adhesion was observed without changes in expression levels of VLA-4 and VLA-5 but was specifically blocked by antibodies against these integrins, thus indicating that overexpression of CrkL activates VLA-4 and VLA-5 to increase the cell adhesion to fibronectin. Studies using CrkL mutants have showed that the N-terminal SH3 domain of CrkL, required for binding C3G, plays a crucial role for integrin activation, because a mutant defective in this domain decreased the cell adhesion to fibronectin. In accordance with this, overexpression of C3G also increased the cell adhesion to fibronectin, whereas a C3G mutant defective in the guanine nucleotide exchange domain significantly inhibited the basal and CrkL-enhanced adhesion. These data indicate that the

CrkL-C3G complex activates VLA-4 and VLA-5 in hematopoietic cells through the guanine nucleotide exchange activity of C3G.

During the preparation of this report, Senechal et al<sup>44</sup> reported that overexpression of CrkL in hematopoietic cells increased adhesion to fibronectin. Senechal et al<sup>44</sup> further showed that individual mutations or deletions of each SH2 and SH3 domain of CrkL abrogated the increase in adhesion. This is at variance with our structure function studies, which showed that the SH2 or C-terminal SH3 domain of CrkL is partially or totally, respectively, dispensable for the increase in cell adhesion and that the N-terminal SH3 domain-defective mutant exerted a dominant negative effect on adhesion. The basis for these discrepancies are unknown but may reflect differences in the structures of CrkL mutants or other experimental conditions. Although Senechal et al<sup>44</sup> suggested the involvement of inte-



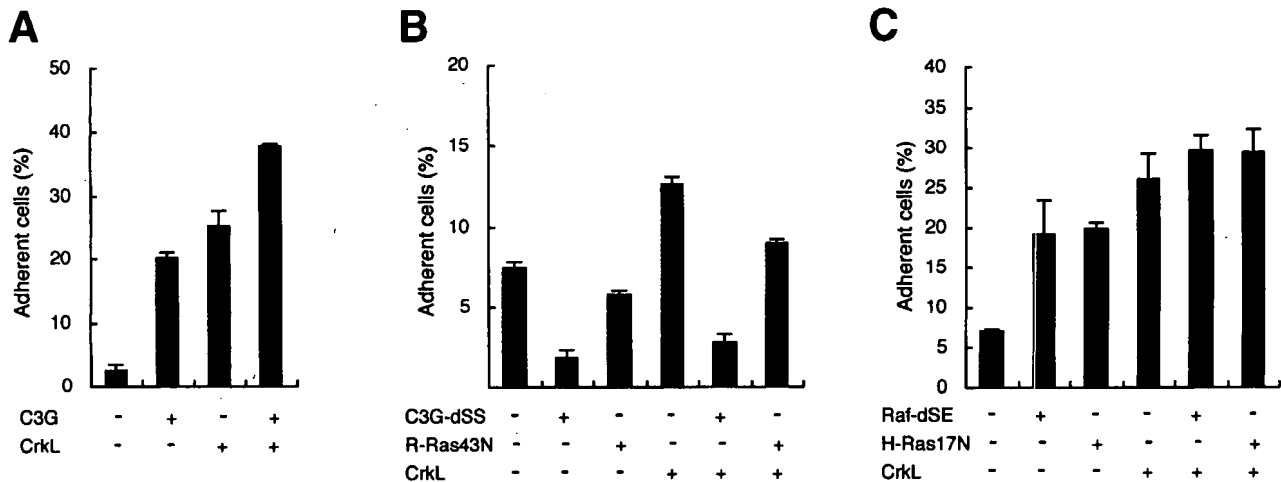
**Fig 4.** The guanine nucleotide exchange domain of C3G is involved in enhancement of cell adhesion. (A) Inducible expression of C3G or its mutant in 32D cells. A clone of 32D/EpoR-Wt cells transfected with pTet-C3G (32DE/Tet-C3G) or pTet-C3G-dSS (32DE/Tet-C3G-dSS), coding for C3G or its mutant lacking the guanine nucleotide exchange domain, respectively, was cultured for 24 hours with (+) or without (-) Tet, as indicated. TCL were extracted and subjected to anti-C3G immunoblotting. Positions of C3G and its mutant, C3G-dSS, are indicated. (B) 32DE/Tet-C3G and 32DE/Tet-C3G-dSS cells were cultured for 24 hours with (+) or without (-) Tet, as indicated, and subjected to the cell adhesion assay as described in Materials and Methods.

grins in CrkL-induced cell adhesion by showing an inhibitory effect of RGD peptides, the integrins involved in adhesion were not identified. The present study, thus, complements and extends that of Senechal et al<sup>44</sup> by showing that VLA-4 and VLA-5, without changes in their expression levels, mediate the CrkL-induced cell adhesion to fibronectin and that CrkL transduces the signal leading to integrin activation through the guanine nucleotide exchange activity of C3G, which is complexed through the N-terminal SH3 domain of CrkL.

Although it has remained to be known how the CrkL-C3G complex activates the signaling pathway leading to integrin

activation through the guanine nucleotide exchange activity of C3G, it is reasonable to speculate that CrkL functions as an adapter protein to recruit C3G to its substrate involved in regulation of the integrin function. In this regard, it is noteworthy that the SH2 domain of CrkL was also required, although partially, for the enhancement of cell adhesion, because previous studies have shown that CrkL binds through its SH2 domain to tyrosine-phosphorylated adhesion-associated proteins, such as paxillin,<sup>45</sup> CAS,<sup>46</sup> HEF-1,<sup>47,48</sup> and Cbl<sup>29,31-33,49,50</sup> in hematopoietic cells expressing BCR/Abl or stimulated with cytokine. In addition, overexpression of CrkL in fibroblasts has been shown to activate many of the same signal transduction pathways as BCR/Abl<sup>51</sup> and to induce tyrosine phosphorylation of paxillin and its association with CrkL.<sup>44</sup> In accordance with these observations, Cbl was constitutively tyrosine phosphorylated and associated with CrkL in 32DE/Tet-CrkL cells withdrawn from tetracycline and starved from cytokine (Y.N., A.A., O.M., unpublished observation, July 1998). Thus, it is possible that overexpression of CrkL induces recruitment of C3G to the vicinity of its substrates involved in regulation of integrin function through interaction between the CrkL SH2 domain and these tyrosine-phosphorylated proteins.

Previously, R-Ras was shown to upregulate the binding affinity of integrins, including VLA-4 and VLA-5 in 32D cells.<sup>38</sup> In contrast, H-Ras or its downstream kinase Raf-1, inhibited the activation of chimeric integrins with multiple  $\alpha$  and  $\beta$  subunit cytoplasmic domains expressed in CHO cells.<sup>43</sup> Although C3G most efficiently activates Rap1/K-Rav1, it also activates R-Ras moderately and H-Ras rather weakly.<sup>52</sup> Therefore, the possible involvement of R-Ras or H-Ras in CrkL-induced activation of cell adhesion was examined in the present study. In accordance with the report by Hughes et al,<sup>43</sup> a dominant negative mutant of H-Ras or that of Raf-1 expressed alone or in combination with CrkL significantly increased the 32D cell adhesion to fibronectin (Fig 5C). Because Rap1/K-Rav1, which C3G activates most efficiently, antagonizes the function of Ras in certain cell lines,<sup>53-55</sup> it is formally possible that the CrkL-C3G complex activates integrins by downregulating the Ras/Raf-1 signaling pathway through activation of Rap1/K-Rav1. However, this possibility is unlikely because overexpression of the CrkL-C3G complex in 32D cells activates the Raf/MAP kinase pathway in 32D cells (A.A., Y.N., O.M., unpublished observation, July 1998). A dominant negative mutant of R-Ras, on the other hand, downregulated the basal as well as CrkL-induced adhesion of 32D/EpoR-Wt cells to fibronectin (Fig 5B), which is in agreement with the previous report.<sup>38</sup> So, it is possible that CrkL may activate VLA-4 and VLA-5 partly through activation of R-Ras. However, the reduction in cell adhesion induced by the dominant negative R-Ras, which was expressed at high levels, was only moderate, thus suggesting that biochemical events involving signaling molecules other than R-Ras should play more important roles in activation of hematopoietic cell adhesion induced by CrkL and C3G. Therefore, to elucidate the signaling pathways mediating the CrkL-induced activation of cell adhesion, further studies are required to analyze the possible modulation of the activities of these Ras family GTPases and to examine the possible involvement of other signaling molecules. The Rho family GTPases, which have also been implicated in enhancing cell adhesion,<sup>56,57</sup>



**Fig 5. Effects of C3G and various mutant signaling molecules on adhesion of 32D cells. (A)** Effects of overexpression of C3G on adhesion of 32D cells to fibronectin. 32D/EpoR-Wt cells were transfected with 30  $\mu$ g of pcDNA-C3G (C3G) and 10  $\mu$ g of pSG-CrkL (CrkL), as indicated, along with 1  $\mu$ g of pRL-SV. The total amount of expression plasmids for each transfection was adjusted to become equal by addition of pcDNA3. Transiently transfected cells were subjected to the cell adhesion assay as described in Materials and Methods. **(B)** Effects of dominant negative mutants of C3G and R-Ras on adhesion of 32D cells to fibronectin. 32D/EpoR-Wt cells were transfected with 40  $\mu$ g of pcDNA-C3G-dSS (C3G-dSS) or pcDNA-R-Ras43N (R-Ras43N) and 5  $\mu$ g of pSG-CrkL (CrkL), as indicated, along with 1  $\mu$ g of pRL-SV. Transiently transfected cells were subjected to the cell adhesion assay. **(C)** Effects of dominant negative mutants of Raf-1 and H-Ras on adhesion of 32D cells to fibronectin. 32D/EpoR-Wt cells were transfected with 35  $\mu$ g of pcDNA-Raf-dSE (Raf-dSE) or pcDNA-H-Ras17N (H-Ras17N) and 5  $\mu$ g of pSG-CrkL (CrkL), as indicated, along with 1  $\mu$ g of pRL-SV. Transiently transfected cells were subjected to the cell adhesion assay.

are of particular interest because v-Crk has recently been reported to activate the Rho-signaling pathway in PC12 cells,<sup>58</sup> although C3G may not directly activate Rho.

CrkL has been implicated in hematopoietic cell signaling from the receptors for Epo, IL-3, thrombopoietin, and stem cell factor, because these factors induce the tyrosine phosphorylation of CrkL and its binding with tyrosine-phosphorylated signaling molecules, including Cbl.<sup>29-33</sup> Intriguingly, these factors also activate the hematopoietic cell adhesion to fibronectin through VLA-4 and VLA-5.<sup>6-9</sup> It is tempting to speculate that CrkL may mediate the signal from these receptors to activate integrins ("inside out" signaling), possibly by recruiting C3G to the vicinity of its effector molecule at the plasma membrane through the binding of CrkL SH2 domain with tyrosine-phosphorylated signaling molecules, such as Cbl. Consistent with this hypothesis, overexpression of CrkL enhanced the Epo- or IL-3-induced cell adhesion as shown in Fig 1D. In addition, it should be noted that, except for the results shown in Fig 1D, all the other cell adhesion assays in the present study were performed under the condition in which cells had been cultured in Epo-containing medium and subsequently allowed to adhere to fibronectin-coated wells for 30 minutes in the presence of IL-3 as described in Materials and Methods. Therefore, the effects of mutants of CrkL or other signaling molecules examined in this study should represent their effects on cytokine-stimulated adhesion of hematopoietic cells. As shown in Fig 1D, overexpression of CrkL also activated the adhesion of cytokine-starved cells. However, this could be explained by the observation that overexpression of CrkL per se induces tyrosine phosphorylation of adhesion-associated proteins and their association with CrkL<sup>44</sup> (Y.N., A.A., O.M., unpublished observation, July 1998). In addition to playing a possible role in the "inside out" signaling, CrkL may also play a role in the

"outside in" signaling, because binding of ligands with integrins or cross linking of integrins also induces tyrosine phosphorylation of signaling molecules, including Hef<sup>48,59</sup> and Cbl<sup>48</sup> and their association with CrkL. Noteworthy in this regard is the observation that the CrkL-overexpressing cells not only showed increased adhesion but also exhibited morphologic changes when attached to culture plate (Fig 1A). Further studies are in progress in our laboratory to examine the effects of CrkL overexpression on the "outside in" signaling pathways as well as on the control of growth, differentiation, apoptosis, motility, and morphologic changes of various hematopoietic cell lines, including those derived from chronic myelogenous leukemia.

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#### REFERENCES

1. Hynes RO: Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69:11, 1992
2. Clark EA, Brugge JS: Integrins and signal transduction pathways: The road taken. *Science* 268:233, 1995
3. Rosenblatt M, Vuillet GM, Leroy C, Coulombel L: Coexpression of two fibronectin receptors, VLA-4 and VLA-5, by immature human erythroblastic precursor cells. *J Clin Invest* 87:6, 1991
4. Williams DA, Rios M, Stephens C, Patel VP: Fibronectin and VLA-4 in hematopoietic stem cell-microenvironment interactions. *Nature* 352:438, 1991
5. Kerst JM, Sanders JB, Slaper-Cortenbach ICM, Doorakkers MCH, Hooibrink B, van Oers RJH, Von dem Borne AEG Kr, van der Schoot Ce: Alpha 4 beta 1 and alpha 5 beta 1 are differentially expressed



during myelopoiesis and mediate the adherence of human CD34+ cells to fibronectin in an activation-dependent way. *Blood* 81:344, 1993

6. Levesque JP, Leavesley DI, Niutta S, Vadas M, Simmons PJ: Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *J Exp Med* 181:1805, 1995

7. Kovach NL, Lin N, Yednock T, Harlan JM, Broudy VC: Stem cell factor modulates avidity of alpha 4 beta 1 and alpha 5 beta 1 integrins expressed on hematopoietic cell lines. *Blood* 85:159, 1995

8. Levesque JP, Haylock DN, Simmons PJ: Cytokine regulation of proliferation and cell adhesion are correlated events in human CD34+ hemopoietic progenitors. *Blood* 88:1168, 1996

9. Gotoh A, Ritchie A, Takahira H, Broxmeyer HE: Thrombopoietin and erythropoietin activate inside-out signaling of integrin and enhance adhesion to immobilized fibronectin in human growth-factor-dependent hematopoietic cells. *Ann Hematol* 75:207, 1997

10. Verfaillie CM, McCarthy JB, McGlave PB: Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. *J Exp Med* 174:693, 1991

11. Miyake K, Weissman IL, Greenberger JS, Kincade PW: Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J Exp Med* 173:599, 1991

12. Papayannopoulou T, Nakamoto B: Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *Proc Natl Acad Sci USA* 90:9374, 1993

13. Yanai N, Sekine C, Yagita H, Obinata M: Roles for integrin very late activation antigen-4 in stroma-dependent erythropoiesis. *Blood* 83:2844, 1994

14. Sugahara H, Kanakura Y, Furitsu T, Ishihara K, Oritani K, Ikeda H, Kitayama H, Ishikawa J, Hashimoto K, Kanayama Y, Matsuzawa Y: Induction of programmed cell death in human hematopoietic cell lines by fibronectin via its interaction with very late antigen 5. *J Exp Med* 179:1757, 1994

15. Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fassler R: Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature* 380:171, 1996

16. Strobel ES, Mobest D, von KS, Dangel M, Ries S, Mertelsmann R, Henschler R: Adhesion and migration are differentially regulated in hematopoietic progenitor cells by cytokines and extracellular matrix. *Blood* 90:3524, 1997

17. Schofield KP, Humphries MJ, de Wynter E, Testa N, Gallagher JT: The effect of alpha4 beta1-integrin binding sequences of fibronectin on growth of cells from human hematopoietic progenitors. *Blood* 91:3230, 1998

18. Yokota T, Oritani K, Mitsui H, Aoyama K, Ishikawa J, Sugahara H, Matsumura I, Tsai S, Tomiyama Y, Kanakura Y, Matsuzawa Y: Growth-supporting activities of fibronectin on hematopoietic stem/progenitor cells *in vitro* and *in vivo*: Structural requirement for fibronectin activities of CS1 and cell-binding domains. *Blood* 91:3263, 1998

19. Mayer BJ, Hamaguchi M, Hanafusa H: A novel viral oncogene with structural similarity to phospholipase C. *Nature* 332:272, 1988

20. Matsuda M, Tanaka S, Nagata S, Kojima A, Kurata T, Shibuya M: Two species of human CRK cDNA encode proteins with distinct biological activities. *Mol Cell Biol* 12:3482, 1992

21. ten Hoeve J, Morris C, Heisterkamp N, Groffen J: Isolation and chromosomal localization of CRKL, a human crk-like gene. *Oncogene* 8:2469, 1993

22. Tanaka S, Morishita T, Hashimoto Y, Hattori S, Nakamura S, Shibuya M, Matuoka K, Takenawa T, Kurata T, Nagashima K, Matsuda M: C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. *Proc Natl Acad Sci USA* 91:3443, 1994

23. Feller SM, Knudsen B, Hanafusa H: Cellular proteins binding to

the first Src homology 3 (SH3) domain of the proto-oncogene product c-Crk indicate Crk-specific signaling pathways. *Oncogene* 10:1465, 1995

24. de Jong R, Haataja L, Voncken JW, Heisterkamp N, Groffen J: Tyrosine phosphorylation of murine CrkL. *Oncogene* 11:1469, 1995

25. Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD, Druker BJ: CrkL is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. *J Biol Chem* 269:22925, 1994

26. ten Hoeve J, Arlinghaus RB, Guo JQ, Heisterkamp N, Groffen J: Tyrosine phosphorylation of CRKL in Philadelphia+ leukemia. *Blood* 84:1731, 1994

27. Nichols GL, Raines MA, Vera JC, Lacomis L, Tempst P, Golde DW: Identification of CRKL as the constitutively phosphorylated 39-kD tyrosine phosphoprotein in chronic myelogenous leukemia cells. *Blood* 84:2912, 1994

28. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J: Tyrosine 207 in CRKL is the BCR/ABL phosphorylation site. *Oncogene* 14:507, 1997

29. Sattler M, Salgia R, Shrikhande G, Verma S, Pisick E, Prasad KV, Griffin JD: Steel factor induces tyrosine phosphorylation of CRKL and binding of CRKL to a complex containing c-kit, phosphatidylinositol 3-kinase, and p120(CBL). *J Biol Chem* 272:10248, 1997

30. Oda A, Miyakawa Y, Druker BJ, Ishida A, Ozaki K, Ohashi H, Wakui M, Handa M, Watanabe K, Okamoto S, Ikeda Y: CrkL is constitutively tyrosine phosphorylated in platelets from chronic myelogenous leukemia patients and inducibly phosphorylated in normal platelets stimulated by thrombopoietin. *Blood* 88:4304, 1996

31. Chin H, Saito T, Arai A, Yamamoto K, Kamiyama R, Miyasaka N, Miura O: Erythropoietin and IL-3 induce tyrosine phosphorylation of CrkL and its association with Shc, SHP-2, and Cbl in hematopoietic cells. *Biochem Biophys Res Commun* 239:412, 1997

32. Gesbert F, Garbay C, Bertoglio J: Interleukin-2 stimulation induces tyrosine phosphorylation of p120-Cbl and CrkL and formation of multimolecular signaling complexes in T lymphocytes and natural killer cells. *J Biol Chem* 273:3986, 1998

33. Barber DL, Mason JM, Fukazawa T, Reedquist KA, Druker BJ, Band H, D'Andrea AD: Erythropoietin and interleukin-3 activate tyrosine phosphorylation of CBL and association with CRK adaptor proteins. *Blood* 89:3166, 1997

34. Miura O, Cleveland JL, Ihle JN: Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. *Mol Cell Biol* 13:1788, 1993

35. ten Hoeve J, Kaartinen V, Fiorctos T, Haataja L, Voncken JW, Heisterkamp N, Groffen J: Cellular interactions of CRKL, and SH2-SH3 adaptor protein. *Cancer Res* 54:2563, 1994

36. Sells MA, Chernoff J: Epitope-tag vectors for eukaryotic protein production. *Gene* 152:187, 1995

37. Bonner TI, Oppermann H, Seeburg P, Kerby SB, Gunnell MA, Young AC, Rapp UR: The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene. *Nucleic Acids Res* 14:1009, 1986

38. Zhang Z, Vuori K, Wang H, Reed JC, Ruoslahti E: Integrin activation by R-ras. *Cell* 85:61, 1996

39. Gossen M, Bujard H: Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89:5547, 1992

40. Chin H, Arai A, Wakao H, Kamiyama R, Miyasaka N, Miura O: Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. *Blood* 91:3734, 1998

41. Kimura M, Takatsuki A, Yamaguchi I: Blastocidin S deaminase gene from *Aspergillus terreus* (BSD): A new drug resistance gene for transfection of mammalian cells. *Biochem Biophys Acta* 1219:653, 1994

42. Chin H, Nakamura N, Kamiyama R, Miyasaka N, Ihle JN, Miura

O: Physical and functional interactions between Stat5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3. *Blood* 88:4415, 1996

43. Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schwartz MA, Ginsberg MH: Suppression of integrin activation: A novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 88:521, 1997
44. Senechal K, Heaney C, Druker B, Sawyers CL: Structural requirements for function of the Crkl adaptor protein in fibroblasts and hematopoietic cells. *Mol Cell Biol* 18:5082, 1998
45. Salgia R, Uemura N, Okuda K, Li JL, Pisick E, Sattler M, de Jong R, Druker B, Heisterkamp N, Chen LB, Groffen J, Griffin JD: CRKL links p210BCR/ABL with paxillin in chronic myelogenous leukemia cells. *J Biol Chem* 270:29145, 1995
46. Salgia R, Pisick E, Sattler M, Li JL, Uemura N, Wong WK, Burky SA, Hirai H, Chen LB, Griffin JD: p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *J Biol Chem* 271:25198, 1996
47. de Jong R, van Wijk A, Haataja L, Heisterkamp N, Groffen J: BCR/ABL-induced leukemogenesis causes phosphorylation of Hef1 and its association with Crkl. *J Biol Chem* 272:32649, 1997
48. Sattler M, Salgia R, Shrikhande G, Verma S, Uemura N, Law SF, Golemis EA, Griffin JD: Differential signaling after beta1 integrin ligation is mediated through binding of CRKL to p120(CBL) and p110(HEF1). *J Biol Chem* 272:14320, 1997
49. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J: Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia. *J Biol Chem* 270:21468, 1995
50. Sattler M, Salgia R, Okuda K, Uemura N, Durstin MA, Pisick E, Xu G, Li JL, Prasad KV, Griffin JD: The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. *Oncogene* 12:839, 1996
51. Senechal K, Halpern J, Sawyers CL: The CRKL adaptor protein transforms fibroblasts and functions in transformation by the BCR-ABL oncogene. *J Biol Chem* 271:23255, 1996
52. Gotoh T, Niino Y, Tokuda M, Hatase O, Nakamura S, Matsuda M, Hattori S: Activation of R-Ras by Ras-guanine nucleotide-releasing factor. *J Biol Chem* 272:18602, 1997
53. Cook SJ, Rubinfeld B, Albert I, McCormick F: RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J* 12:3475, 1993
54. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M: A ras-related gene with transformation suppressor activity. *Cell* 56:77, 1989
55. Sakoda T, Kaibuchi K, Kishi K, Kishida S, Doi K, Hoshino M, Hattori S, Takai Y: smg/rap1/Krev-1 p21s inhibit the signal pathway to the c-fos promoter/enhancer from c-Ki-ras p21 but not from c-raf-1 kinase in NIH3T3 cells. *Oncogene* 7:1705, 1992
56. Laudanna C, Campbell JJ, Butcher EC: Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science* 271:981, 1996
57. Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV: Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 390:632, 1997
58. Altun-Gultekin Z, Chandriani S, Bougeret C, Ishizaki T, Narumiya S, de Graaf P, van Bergen en Henegouwen P, Hanafusa H, Wagner JA, Birge RB: Activation of Rho-dependent cell spreading and focal adhesion biogenesis by the v-Crk adaptor protein. *Mol Cell Biol* 18:3044, 1998
59. Manic SN, Beck A, Astier A, Law SF, Canty T, Hirai H, Druker BJ, Avraham H, Haghighyehi N, Sattler M, Salgia R, Griffin JD, Golemis EA, Freedman AS: Involvement of p130(Cas) and p105(HEF1), a novel cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human B cells. *J Biol Chem* 272:4230, 1997

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Regular Article

# Human Neural Precursor Cells Express Low Levels of Telomerase *in Vitro* and Show Diminishing Cell Proliferation with Extensive Axonal Outgrowth following Transplantation

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## Abstract

Worldwide attention is presently focused on proliferating populations of neural precursor cells as an *in vitro* source of tissue for neural transplantation and brain repair. However, successful neuroreconstruction is contingent upon their capacity to integrate within the host CNS and the absence of tumorigenesis. Here we show that human neural precursor cells express very low levels of telomerase at early passages (less than 20 population doublings), but that this decreases to undetectable levels at later passages. In contrast, rodent neural precursors express high levels of telomerase at both early and late passages. The human neural precursors also have telomeres (approximately 12 kbp) significantly shorter than those of their rodent counterparts (approximately 40 kbp). Human neural precursors were then expanded 100-fold prior to intrastriatal transplantation in a rodent model of Parkinson's disease. To establish the effects of implanted cell number on survival and integration, precursors were transplanted at either 200,000, 1 million, or 2 million cells per animal. Interestingly, the smaller transplants were more likely to extend neuronal fibers and less likely to provoke immune rejection than the largest transplants in this xenograft model. Cellular proliferation continued immediately post-transplantation, but by 20 weeks there were virtually no dividing cells within any of the grafts. In contrast, fiber outgrowth increased gradually over time and often occupied the entire striatum at 20 weeks postgrafting. Transient expression of tyrosine hydroxylase-positive cells within the grafts was found in some animals, but this was not sustained at 20 weeks and had no functional effects. For Parkinson's disease, the principal aim now is to induce the dopaminergic phenotype in these cells prior to transplantation. However, given the relative safety profile for these human cells and their capacity to extend fibers into the adult rodent brain, they may provide the ideal basis for the repair of other lesions of the CNS where extensive axonal outgrowth is required.

**Author Keywords:** neural precursor cells; neural progenitor cells; neural stem cells; neural transplantation; telomerase; telomeres; human; cell division; immune rejection

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